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The yeast ADP/ATP carrier. Mutagenesis and second-site revertants

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Abstract

Results of mutagenesis and selection of spontaneous second-site revertants of the yeast ADP/ATP carrier AAC2 is described. Currently, 50 mutants have been made in AAC2 at 35 locations. Yeast carrying mutations at K38, K48, R96, D149, R152, R204, D249, R252, R253, R254 and R294 are all unable to grow on glycerol. Seven of these mutants have yielded second-site revertants when plated on rich yeast media containing glycerol and ethanol. The R96 mutants and the R254 and R253 mutants produce similar changes in the AAC2 molecule because the same sites are affected by their revertant mutations. This system of mutations and revertants is now poised to yield insights into the dynamics of ADP and ATP transport, and mitochondrial carrier structure in general.

Keywords: AAC2; Mitochondrial carrier; ADP/ATP carrier; Second-site revertant

1. Introduction

The rapid progress in sequencing the yeast genome will soon allow us to identify all the mitochondrial carriers in a single-celled organism. Even now, at the end of 1995, 70% of the yeast genome is in Genbank and 31 open reading frames appear to be mitochondrial carriers. This extrapolates to 44 carriers in the complete genome. There is a significant degree of redundancy in yeast, so 44 genes does not imply 44 functions. Analysis of random lacZ insertions by Burns et al. [1] showed that only 25% of gene disruptions had a detectable phenotype. This will probably be true for the carrier genes as well. The ADP/ATP carrier is represented by three genes, AAC1, AAC2 and AAC3. Only AAC2 is normally expressed. The phosphate carrier MIR1 has another silent partner YER053c that is 38% identical in amino acid sequence. This is greater than the percent identity between yeast and mammalian phosphate carriers so the YER053c gene can be assumed to be a phosphate carrier as well. Since the knockout of MIR1 is unable to grow on non-fermentable carbon sources [2], YER053c is not functionally redundant under normal circumstances. The two genes MRS3 and MRS4 that are high copy suppressors of mitochondrial splicing defects [3] are clearly isoforms (75% identical), as are YMC1 [4] and YMC2 (Genbank Z35973) with 69% identity. The two genes YEL006w (Genbank U18530) and YIL006w (Genbank Z38113) are 58% identical and probably have the same function. Therefore, of the 31 known carriers in yeast at least five are redundant (16%). This analysis leads to an estimate of about 37 unique functions among the 44 carriers predicted to exist in yeast. This number is three times the known functions attributed to mitochondrial carriers by LaNoue and Schoolwerth [5], and more than twice the number given by Palmieri [6]. Of the 31 carriers identified by sequence homology in yeast only four functions have been determined (the ADP/ATP carrier, the citrate carrier [7], the phosphate carrier [8] and the FAD carrier, FLX1 [9,10]) accounting for only seven of the sequences. So far, the α-ketoglutarate carrier has not been identified in yeast and the uncoupler protein is strictly mammalian.

Clearly, one of the major undertakings in mitochondrial carrier research will be to discern what the normal substrates are for this moderately sized protein family. The problem of going from sequence to function has recently been reviewed by Oliver, with special reference to yeast [11]. The first step along this path will be the construction of knockouts of all these genes to see what if any phenotype they have. So far, 14 yeast carriers have been disrupted, but only five are unable to grow on glycerol. Of

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these, PET8 [12] and RIM2 [13] still have no defined substrates. The more direct approach would be to express all of the carriers in *Escherichia coli* and screen them for activity in a series of reconstitution assays. This approach could succeed in identifying those functions that are known to be in yeast, but cannot be identified by sequence alone. The reconstitution method has been tried with the citrate carrier [7] and the phosphate carrier [14], though efforts to express AAC2 in *E. coli* have not succeeded. Some potential pitfalls of this strategy are codon bias differences between yeast and *E. coli*. This is a particular problem for the arg codons AGA and AGG and the leu codon CGA. AGA and AGG are the most common codons for arginine in yeast making up 69% of the Arg codon usage. In *E. coli*, they are among the rarest of codons, comprising only 7% of arg codon usage. CGA is also very rare in *E. coli*, at just 3% of leu codons. In yeast CGA is 14%. There are 19 of these undesirable codons in AAC2, including a run of three AGA codons in a row at the conserved Arg triplet.

Perhaps more important than defining function for these carriers will be the continuing task of figuring out how they transport their substrates across the mitochondrial inner membrane. This will depend on a handful of carriers that can be isolated in large quantity from mammalian tissue, or that have defined systems for mutagenesis and expression in yeast or *E. coli*. These include AAC2, the major isoform of the yeast ADP/ATP carrier, the mammalian heart AAC, the uncoupler protein that is now expressed in yeast [15,16], the phosphate carrier and the citrate carrier. For reviews of mitochondrial carrier structure, function and evolution, see Refs. [6,17–21].

2. Mutagenesis of the yeast AAC2

Fig. 1 shows the residues of the AAC2 that have been mutated in our laboratory [18,22]. The six transmembrane segment model has only four charged amino acids in the membrane region. These are K38, R96, R204 and R294. All of these residues have been modified and all mutants are unable to grow on glycerol/ethanol plates. These are referred to as gly$^-$ mutants. In addition to these conspicuous charges, the triple arginine sequence in the third domain (252–254) has also been modified and all three of these charges are also required for growth on glycerol.

The molecule is drawn to emphasize the three-domain structure of the protein. In this structure, certain features are repeated in the AAC2 and indeed in all the ADP/ATP carriers with known sequence (about 30 sequences). An alignment of 119 mitochondrial carriers or 31 yeast carriers as of March 1, 1996 is available on the World Wide Web at http://drnelson.utmem.edu/homepage.html. For a select number of positions, this conservation of structure extends to nearly all the carrier sequences, independent of substrates transported. These residues include the negative charges at the bottom of the first, third and fifth helices,

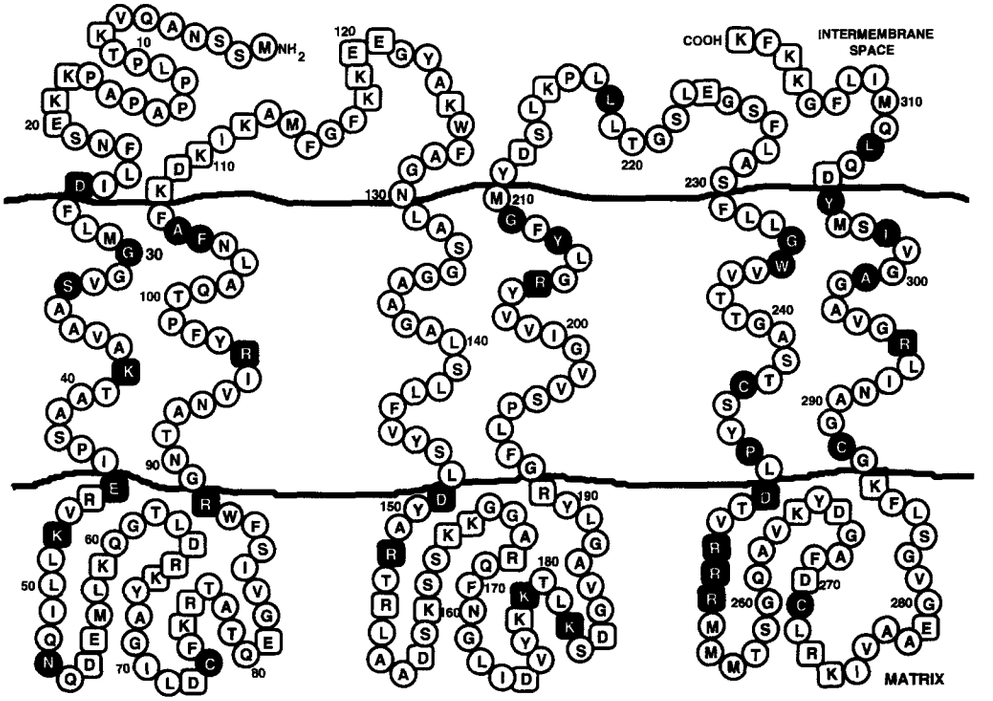

Fig. 1. Location of mutants in the yeast AAC2. K38A, K48A, C73S, R88[A,D], R96[A,D,H,L,P,T], D149[G,S], [R152A + L218S], K179M, [K179I + K182I], R204L, W235F,C244S, P247G, D249S, R252I, R253I, R254I, C271S and R294A were made by site-directed mutagenesis. D26[E,V], G30[C,V], S33[I,N], E45[G,Q], N53I, F105[L,V], A106T, R152K, Y207H, G209S, G234S, W235L, R252T, C288Y, A299S, I302T, Y305H, and L308P arose as second-site revertants. Many of these are described in more detail in Refs. [17,18,22,27].

and the positive charges two amino acids below them. We have mutated all six of these charges in AAC2 and find that five mutants are gly$^-$. Only E45 does not seem to be required.

Not all of the charged residues we have mutated are critical for function. The aspartate at position 26 is not required, and the conserved R88 at the bottom of the second helix is also dispensable. K179 and K182, labeled by pyridoxal phosphate from the outside surface [23], and thought to penetrate deep into the membrane, can also be neutralized without serious consequences.

All four cysteines in AAC2 have been modified. C73, C244, C271 and C288 are all non-essential for growth on glycerol. Work is now under way to mutate all of these residues in the same molecule to make a cysteine free protein. Such a molecule could have cysteine added back at any desired location, as long as that site was not essential. The usefulness of such constructs would be invaluable in helix contact mapping and for introduction of specific labels into the protein.

Fifteen other sites in AAC2 have been mutated, either directly or through selection of second site revertants (see below). All of these mutants are gly$^+$, although many of them do not exist as single mutants, but only in conjunction with another gly$^-$ mutant used for selection of revertants. The proline at 247 was mutated to glycine without affecting growth on glycerol. This was surprising because the prolines at the bottom of the first third and fifth helices are conserved in nearly all mitochondrial carriers. Serine is found in plants and lower eukaryotes in domain two, but otherwise, replacement of the proline is extremely rare. Only two cases are seen and these are both from systematic sequencing of the yeast genome. Chromosome XII (U19028.14) and chromosome XIII (Z48756.3) have phe and thr respectively in domain two, though this may be due to sequencing errors. Glycine never occurs at this location in 119 carriers, though the lack of a side chain may make folding at this position very flexible and lead to the correct structure even without the proline.

3. Second-site revertants

All of the gly$^-$ mutants of AAC2 involve loss of a charge, either positive or negative. Neutral residues tested so far have not been essential for activity when mutated. All of the glycerol-negative mutants have been used to select second-site revertants. Not every mutant has yielded revertants. Those that do tend to fall into one of two main categories. Either they are intragenic or extragenic. The intragenic revertants are much more common. Only a few extragenic mutants are available and these have not been characterized. The existence of extragenic mutants strongly suggests that another protein or proteins interact with the ADP/ATP carrier in the membrane. One candidate for such an interaction is the F_0 portion of the ATP synthase. The F_0 has been shown to be involved with AAC2 by a series of experiments involving fluorescence quenching of pyranine by oligomycin [24]. The quenching can be blocked or reversed by adding atractyloside or bongkrekic acid, specific inhibitors of AAC2. Bongkrekic acid does not affect oligomycin-dependent quenching in strains of yeast lacking AAC2. We are in the process of sequencing subunit 9 and subunit 6 of the ATPase from our extragenic revertants to see if they might be the sites of these revertant mutations.

The intragenic mutants can be point mutants or recombination products with the AAC3 gene [25]. This gene is highly similar to AAC2 and it has not been deleted from the genome of our host yeast. Occasionally we find our gly$^-$ mutants have recombined with AAC3 to recover the wild-type sequence at the mutation, plus some AAC3 sequence on either side. These are not of much interest to us and eventually we will delete the AAC3 gene to prevent their happening at all. The point mutants can be same-site or second-site revertants. Same-site revertants are selected against by making a double or triple nucleotide change in the original mutation. This requires multiple changes to go back to the wild-type sequence. Still, this type of same-site revertant does occur. For example, a revertant of R152A resulted in A152K, restoring the positive charge with a different amino acid at this site.

The really interesting revertants are second-site revertants. These also fall into two categories. Either they involve mutation of neutral residues to other neutral residues, or they result in loss of a complementary charge. These latter mutants imply that a charge pair may exist between the two sites in the wild-type protein. We have never seen a revertant yet that introduced a new charge at a second site, though that was the original expectation for the intrahelical R294A revertants. If a charge was removed from a transmembrane helix, it made sense that the same charge would reappear on an adjacent transmembrane helix by selection for revertants. This phenomenon was observed for mutants of the DCCD binding subunit of the bacterial F_1F_0 ATPase [26]. A negative charge was removed and a new negative charge appeared on the second transmembrane helix of this small protein. When the R294A mutant was selected on YPGE plates, three independent revertants all showed loss of the negative charge at E45, either to gln or gly. The E45G or E45Q mutants were gly$^+$ when separated from their parent mutation R294A. This made it impossible to select backwards for the mutation of R294 starting with the E45 mutants. For a more detailed account of the biochemistry involved in the R294A mutant see Refs. [17,27]

One interesting feature of AAC2 is the homology between domains. R294 is homologous to position 96 in the second transmembrane helix, and both sites contain arginine. Furthermore, E45 is homologous to D149, both with a negative charge. There was a chance that R96 and D149 were in another charge pair, and this might be detectable

by selection of second-site revertants starting with mutants of R96. We actually had six different mutants of R96 available, (A, D, H, L, P and T) and all were gly$^-$. Some of these were not suitable for selection, because arginine could reappear with one nucleotide change. After many attempts to find second-site revertants, R96L gave S33I and R96A gave three revertants, D26V, C288Y and Y305H. None of the revertants involved D149.

The data were suggestive that there was not a charge pair between R96 and D149 even though they were homologous to E45 and R294. Apparently, though the molecule has three-fold homology, there is not strict symmetry between all of the domains. The occurrence of D26V might suggest a charge interaction between R96 and D26, but this is unlikely because the two are quite far apart and there are three other revertants of R96 mutants that do not involve charges. It is more probable that D26V has more to do with a geometric change at a particular location than with a charge interaction between D26 and R96. The revertants of R96 are far apart in the sequence and on opposite sides of the membrane from each other. The interpretation of these mutations is not clear.

The R96 revertants just mentioned are revertants involving mainly neutral residues changed into other neutral residues. There is another large group of these kind of revertants. They were selected starting from mutants of the arg triplet [22]. The sequence RRRMMM at 252–257 in AAC2 is highly conserved among ADP/ATP carriers. Each Arg was mutated to ile separately and the gly$^-$ mutants were selected for regain of function on glycerol/ethanol plates. R252I never gave any revertants. R253I gave four and R254I gave 11. These results show a strong influence of sequence position on the ability to revert. The three arginines, though adjacent in the sequence, are clearly under different constraints imposed by the function of the protein. R252 seems to be indispensable or nearly so, while mutations at R254 are not nearly so unsalvagable.

One unexpected feature of the 15 revertants was their location. Only N53I was on the matrix side of the membrane with the arg triplet. All the others were either on the cytoplasmic side or they were in the membrane near the cytoplasmic side. All revertants were distant from the arg triplet in the sequence. It is likely that N53 is involved in a polar interaction with R253, but the other residues were probably not in physical contact with the arg triplet. We speculated that the geometry on the far side of the membrane was altered by mutation of the arginines, through a shift in the bottom of helix 5. The revertant mutations were able to restore this defect in a variety of ways.

Some of the revertants from the arg triplet mutants were at identical positions to revertants of R96 mutants. Y305H occurred starting from R96A and R253I. S33I was found starting from R96L and S33N was found starting with R254I. D26E was a revertant of R254I and D26V was a revertant of R96A. This additional mutant at D26 supports the argument made earlier that the D26V mutant does not indicate a charge pair with R96. The fact that the same sites are being affected by two very different mutations, leads to a prediction that both R96 and the arg triplet are interacting with a common third site. Presumably, this third site would be a negatively charged residue. The disruption of this common interaction could then be responsible for the similar alteration of the AAC2 molecule. D149 was already suggested as a possible interacting residue with R96 based on homology to E45 and R294. However, no evidence from revertants supported this idea. To confirm or deny such a possibility, double mutants were constructed with changes at both R96 and D149. These were all gly$^-$. When the double mutant R96T and D149G was selected for revertants, one revertant was found. It was G30V. G30C was previously shown to be a revertant of R254I, which adds to the evidence that R96 mutants and arg triplet mutants have similar effects on AAC2 structure. It is surprising to us that a single change at G30 could resurrect the double mutant of R96T and D149G. Both of these mutants alone are gly$^-$. The mutants that are shared revertants coming from both R96 and the arg triplet are all found in a limited area at the top of transmembrane segments 1 and 6. These two helices are probably adjacent in light of the E45-R294 charge pair. The significance of this localized effect is not known and cannot be known unless more details of the AAC2 structure can be determined.

4. Future directions

The selection for second-site revertants has been very successful so far and we will continue to exploit it. A phrase often heard in Mike Douglas' lab was "Let the protein talk to you." The selection process has already yielded an interesting collection of charge pair revertants in addition to the R294-E45 pair, but these cannot be discussed more fully here.

In the absence of a crystal structure, the understanding of mitochondrial carrier structure will depend on building up a detailed set of structural constraints such as charge pairs and perhaps engineered disulfide bonds. We are actively moving into this phase of research now. Of course the nature of the dimer interaction needs to be clarified and this question may also be resolved using a combination of genetics and biochemistry.

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References

[1] Burns, N. Grimwade, B., Ross-MacDonald, P.B., Choi, E.-Y., Finberg, K., Roeder, G.S. and Snyder, M. (1994) Genes and Dev. 8, 1087–1105.

[2] Murakami, H., Blobel, G. and Pain, D. (1990) Nature 347, 488–491.

[3] Wiesenberger, G., Link, T.A., von Ahsen, U., Waldherr, M.and Schweyen, R.J. (1991) J. Molec. Biol. 217, 23–37.

[4] Graf, R., Baum, B. and Braus, G.H. (1993) Yeast 9, 301–305.

[5] LaNoue, K.F. and Schoolwerth, A.C. (1979) Annu. Rev. Biochem. 48, 871–922.

[6] Palmieri, F. (1994) FEBS Lett. 346, 48–54.

[7] Kaplan, R.S., Mayor, J.A. Gremse, D.A. and Wood, D.O. (1995) J. Biol. Chem. 270, 4108–4114.

[8] Phelps, A., Schobert, C.T. and Wohlrab, H. (1991) Biochemistry 30, 248–252.

[9] Tzagoloff, A.A., Jang, J. Glerum, D.M. and Wu, M. (1996) Mol. Cell. Biol., in press.

[10] Wu, M., Repetto, B. Glerum, D.M. and Tzagoloff, A. (1995) Mol. Cell. Biol. 15, 264–271.

[11] Oliver, S.G (1996) Nature 379, 597–600.

[12] Lalo, D., Stettler, S. Mariotte, S. Gendreau, E. and Thuriaux, P. (1994) Yeast 10, 523–533.

[13] Desmolis, N., Mallet, L., Bussereau, F. and Jacquet, M. (1993) Yeast 9, 645–659.

[14] Wohlrab, H. and Briggs, C. (1994) Biochemistry 33, 9371–9375.

[15] Arechaga, I., Raimbault, S., Prieto, S., Levi-Meyrueis, C., Zaragoza, P., Miroux, B., Ricquier, D., Bouillaud, F. and Rial, E. (1993) Biochem. J. 296, 693–700.

[16] Bouillaud, F., Arechaga, I., Petit, P.X., Raimbault, S., Levi-Meyrueis, C., Casteilla, L., Laurent, M., Rial, E. and Ricquier, D. (1994) EMBO J. 13, 1990–1997.

[17] Klingenberg, M. and Nelson, D.R. (1995) in Biochemistry of Cell Membranes (Papa, S. and Tager, J.M., eds.), Birkhäuser, Basel, Switzerland, pp. 191–219.

[18] Nelson, D.R., Lawson, J.E., Klingenberg, M. and Douglas, M.G. (1993) J. Molec. Biol. 230, 1159–1170.

[19] Krämer, R. and Palmieri, F. (1989) Biochim. Biophys. Acta 974, 1–23.

[20] Walker, J.E. and Runswick, M.J. (1993) J. Bioenerg. Biomembr. 25, 435–446.

[21] Kuan, J. and Saier, M.H., Jr. (1993) Crit. Rev. Biochem. Molec. Biol. 28, 209–233.

[22] Nelson, D.R. and Douglas, M.G. (1993) J. Molec. Biol. 230, 1171–1182.

[23] Bogner, W., Aquila, H. and Klingenberg, M. (1989) Eur. J. Biochem. 161, 611–620.

[24] Ziegler, M. and Penefsky, H.S. (1993) J. Biol. Chem. 268, 25320–25328.

[25] Kolarov, J., Kolarova, N. and Nelson, N. (1990) J. Biol. Chem. 265, 12711–12716.

[26] Miller, J.M., Oldenburg, M. and Fillingame, R.H. (1990) Proc. Nat. Acad. Sci. USA 87, 4900–4904.

[27] Klingenberg, M. and Nelson, D.R. (1994) Biochim. Biophys. Acta 1187, 241–244.